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(54) Title: ISOLATION AND AMPLIFICATION OF NUCLEIC ACID MATERIALS

(57) Abstract

The invention relates to methods for easily separating single stranded nucleic acid material from double stranded nucleic acid material in a sample containing both. By the right choice of at least one chaotropic agent, preferably a guanidine salt, at a selected concentration and other suitable conditions such as chelating agents, pH and the like, it is possible to bind double stranded material to a solid phase such as silica particles, whereas single stranded material will not bind under those circumstances. By separating the silica particles from the sample the double stranded nucleic acid material is removed. It can easily be eluted from the silica particles. In a second step the single stranded material may be bound to a solid phase by selecting a different set of conditions. The particles can again be separated from the sample and the single stranded material may now be eluted. For every efficient separations, the process may be repeated. Following the separation of the two kinds of nucleic acid, either kind may be amplified. Methods of amplification are provided which do not need sequence data of the material to be amplified. In these methods a primer will be provided with an amplification motif and a random hybridization motif.

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ISOLATION AND AMPLIFICATION OF NUCLEIC ACID MATERIALS

The invention relates to the field of purification and amplification of nucleic acids from nucleic acid containing starting materials, especially from biological materials such as urine, faeces, sperm, saliva, whole blood, serum or other body fluids, fractions of such fluids such as leucocyte fractions (buffy coats), cell cultures and the like, but also samples from the environment such as soil, water and the like.

Until recently isolation and/or purification of nucleic acids from complex mixtures as described above was a laborious, multi-step procedure. In EP 0389063, incorporated herein by reference, a simple and rapid purification of nucleic acid material from a complex mixture is disclosed. This procedure comprises treating the complex mixture, such as whole blood with a chaotropic agent in the presence of a nucleic acid binding silica solid phase material under conditions that allow for binding of all nucleic acid material to said solid phase and separating said solid phase from the mixture. The reference shows that both single stranded and double stranded nucleic acids are bound to the solid phase if present in a mixture. The reference also discloses amplification (PCR) of a certain nucleic acid with a known sequence, suspected to be present in a mixture.

Thus said reference teaches a simple and rapid detection method for known nucleic acids suspected to be present in a sample.

In many cases the nature of the target nucleic acid (double atranded or single stranded) may not be known beforehand, or there may be many different targets necessary to be analyzed. In these cases the rapid but rather crude method described above may not be sophisticated enough and further separations of the crude material may be wanted. Fractionation of mixtures of double- (ds) and single-stranded (ss) nucleic acids (NA) into single- and double-stranded forms is frequently needed e.g. in the separation of labelled ss-NA

probes from ds-hybrids, in the separation of in vitro transcripts from ds-DNA templates, and in the separation of genomic DNA from mRNA. Currently, the separation of different kinds of nucleic acids can be accomplished by several techniques. Electrophoresis can be used to fractionate different forms of nucleic acids, because of differences in size and shape (1-3). Centrifugation takes advantage of differences in density (4), and more recently the technology of high-performence liquid chromatography (HPLC) has been applied to separate and purify single- and double-stranded DNA and RNA molecules (5-8).

RNA purified from eukaryotic cells by the currently most widely used procedure (9) appears to contain significant amounts of genomic DNA, an adaptation which reduces genomic DNA contamination of the ss-RNA fraction has recently been described (10).

It is not possible to look at single stranded and/or double stranded material separately using the method of EP 0389063 because the method does not discriminate between the two.

The present invention therefor provides a method for separating single stranded nucleic acid material from double stranded nucleic acid material comprising contacting a mixture of the both with a liquid comprising a chaotropic agent and a nucleic acid binding solid phase, whereby the liquid has a composition such that double stranded nucleic acid binds to the solid phase and a substantial amount of single stranded nucleic acid does not and separating the solid phase from the liquid. Suitable circumstances to arrive at such a separation can be determined by the person skilled in the art.

Circumstances under which double stranded material binds to the solid material and single stranded material will vary, however important parameters to obtain such differential binding are the concentration of the chaotropic agent, which should roughly be between 1-10 M, preferably between 3-6 M and particularly about 5 M; the concentration of chelating agent, which in the case that EDTA is applied should be equal to or

greater than 10 mM and preferably not higher than 1 M; the pH of the aqueous solution in which the separation is carried out should be above 2 when a thiocyanate is used as chaotropic agent and it should be below 10 because otherwise there is a risk that the ds material will become ss. The temperature at which the process is carried out seems to be non-critical, however, it is probably best to keep it between 4°C and 60°C. An important aspect of the process is of course that the ds material remains double stranded during the separation. Under the circumstances as disclosed above this will normally be the case if the ds nucleic acid is at least 50 bp long at 40% GC basepairs. The skilled artisan knows how this length may vary with lower or higher GC content. In Van Ness et al (26) and/or Thompson et al (27) it is shown that the whole process depends on intricate interactions between a.o. the factors mentioned above. Using this disclosure and the cited references the skilled artisan will be able to adjust the circumstances to his or her particular process.

Chaotropic agents are a very important feature of the present invention. They are defined as any substance that can alter the secondary, tertiary and/or quaternary structure of nucleic acids. They should have no substantial effect on the primary structure of the nucleic acid. If nucleic acids are present associated with other molecules, such as proteins, these associations can also be altered by the same or different chaotropic agents.Many chaotropic agents are suitable for use in the present invention, such as sodium iodide, potassium iodide, sodium (iso)thiocyanate, urea or guanidinium salts, or combinations thereof. A preferred class of chaotropic agents according to the invention are guanidinium salts, of which guanidinium thiocyanate is most preferred.

By serendipity we found that ss-nucleic acid did not bind to silica particles or diatomeous earth in the presence of buffer L11 (see examples), whereas ds nucleic acid did. Experiments with different circumstances showed that addition of Mg^{2+} or other positive (bivalent) ions to the unbound

fraction was of great importance. The best results were obtained with a concentration of bivalent ion (Mg^{2+}) about equal to the concentration of the chelating agent (EDTA).

The solid phase to be used is less critical. Important is that it should bind nucleic acids reversibly.

Many such materials are known, of which a number are silicium based, such as aluminium silicate and the like, preferably silica. Silica is meant to include SiO_2 crystals and other forms of silicon oxide, such as diatom skeletons, glass powder and/or particles and amorphous silicon oxide. The solid phase may be present in any form, it may even be the vessel which contains the nucleic acid mixtures or a part of such a vessel. It may also be a filter or any other suitable structure. Apart from silicium based materials other materials will also be suitable, such as nitrocellulose (filters), latex particles and other polymeric substances. A preferred form of the solid phase is a particulate form, which allows for easy separation of bound and free material, for instance by centrifugation. The particle size of the solid phase is not critical. Suitable average particle sizes range from about 0.05 to 500 µm. Preferably the range is chosen such that at least 80, preferably 90 % of the particles have a size between the values just mentioned. The same holds true for the preferred ranges of which the average particle sizes are between 0.1 and 200 µm, preferably between 1 and 200 µm. The binding capacity of a given weight of the particles increases with decreasing size, however the lower limit of the size is when particles cannot easily be redispersed after separation through for instance centrifugation. This will be the case in starting material rich in nucleic acids containing many nucleic acids of a higher molecular weight. The particles and the nucleic acids may form aggregates in these cases. The person skilled in the art will be able to choose the right particle size for the particular application envisioned. The formation of aggregates may be avoided by using fractionated silica or diatomaceous earth in a number of applications.

A further embodiment of the present invention is a method for isolating single stranded nucleic acid material from a mixture of nucleic acid material, comprising the steps of subjecting the mixture to a method as described hereinabove and treating the supernatant containing the single stranded nucleic acid material with a second liquid comprising a chaotropic agent and a second nucleic acid binding solid phase, whereby the second liquid has a compositon such that the resulting mixture of supernatant and second liquid allow for binding of the single stranded nucleic acid material to the second solid phase.

This way the double stranded nucleic acid material is removed from the crude mixture and the single stranded nucleic acid is purified from the remaining still crude mixture in another single step. Both the double stranded material and the single stranded material are reversibly bound to the respective solid phases, so that they may be easily eluted from said solid phases to undergo further analysis or other treatments. A very useful further treatment is the amplification of the (double or single stranded) nucleic acid material.

Both types can be amplified, or both types may be converted into one another so that they can be amplified. The present invention provides in yet another embodiment a method for amplifying single stranded nucleic acid material comprising the steps of hybridizing the single stranded nucleic acid with primers and elongating the probes using an enzyme which adds nucleotides to the primer sequence using the hybridized single strand material as a template, whereby at least one primer comprises a random hybridizing sequence and an amplification motif.

Single-stranded nucleic acids purified in accordance with the invention were used as input for a cDNA synthesis reaction using primers with random 3' ends (tagging primers) for the first and second strand synthesis (see the outline in Fig. 7).

These tagged cDNAs are then amplified by using only one PCR primer homologous to the PCR motif present in both tagging

primers. The tagging primer used in the first strand synthesis (TAG 20) has been especially designed to facilitate subsequent direct sequencing of the resultant PCR products.

In contrast with most other protocols (16-22) the described method does not need any sequence data at all, and the majority of amplified products can be visualized on ethidiumbromide stained agarose gels as discrete bands, which makes isolation and direct sequencing of the amplified cDNA feasible. The criteria for amplification are well known in the art. The length of suitable primers, suitable buffers, suitable melting temperatures for separating strands, suitable hybridization conditions can all be determined using standard handbooks in the field.

Of course the sequences which are exemplified can be varied without departing from the present invention. It is not so much important what sequence is used as an amplification motif, as long as it is suitable for hybridization and primer extension purposes. Suitable limits depend on the conditions which can be varied by the person skilled in the art. Usually primers will be at least 10 bases long and not much longer than 100 bases.

The amplification embodiments of the invention are exemplified using PCR (polymerase chain reaction). Other amplification methods are of course equally suitable.

The exemplified label (or tag) on the primers is DIG (digoxygenin). However other labels are available and well known in the art.

The invention will now be explained in further detail in the following detailed description.

Separation / Isolation

MATERIALS AND METHODS

Source of nucleic acids.

Phage MS-2 ss-RNA (3569 nt), E. coli rRNA (16 and 23S; 1,7kb and 3,5kb respectively), phage M13 ss-DNA (7599 nt) and

HindIII digested phage lambda ds-DNA were purchased from Boehringer (Mannheim, Germany). Rotavirus ds-RNA was purified from feces of an infected individual by protocol Y/SC (11). Plasmid DNA was purified from E. coli HB101 as described by Ish-Horowicz and Burke (13) followed by column chromotography with Sepharose CL2B (Pharmacia, Inc. Uppsala, Sweden). Total NA was purified from E.coli by protocol Y/D (11).

Chemicals.

Guanidiniumthiocyanate (GuSCN) was obtained from Fluka (Buchs, Switzerland).

EDTA (Titriplex) and MgC12.6H20 were obtained from Merck (Darmstadt, Germany). TRIS was obtained from Boehringer (Mannheim, Germany). The preparation of size-fractionated silica particles (silica coarse, SC) and diatom suspension has been described (11). Triton X-100 was from Packard (Packard Instrument Co., Inc., Downers Grove, Ill).

Composition of buffers.

The lysis/binding buffer L6, washing buffer L2, and TE (10mM Tris.HCI, 1 mM EDTA; pH=8.0) have been described (11). 0.2M EDTA (pH 8.0) was made by dissolving 37.2 g EDTA (Merck, Germany) and 4.4 g NaOH (Merck, Germany) in aqua in a total volume of 500 ml. Lysis/binding buffer L11 was made by dissolving 120 g of GuSCN in 100 ml 0.2M EDTA (pH=8.0). Binding buffer L10 was prepared by dissolving 120 g GuSCN in 100 ml 0.35M TRIS.HCl (pH 6.4); subsequently 22 ml 0.2M EDTA (pH 8.0) and 9.1 g Triton X-100 were added and the solution was homogenized; finally 11 g of solid $MgCl_2.6H_20$ was added. The final concentration of $MgCl_2$ in L10 is about 0.25M. L10 is stable for at least 1 month when stored at ambient temperature in the dark.

Fractionation of ds-NA and ss-NA by protocol R.

Th procedure is outlined in Figure 1. A 50µl specimen (containing a mixture of NA-types in TE buffer) was added to a mixture of 900µl L11 and 40µl SC in an Eppendorf tube and was

subsequently homogenized by vortexing. After 10 min. binding at room temperature, the tube was centrifuged (2 min. at approx. 10.000 x g) which resulted in a silica/ds-NA pellet ("initial silica pellet") and a supernatant containing ss-NA.

To recover ss-NA forms (protocol R-sup), 900µl of the supernatant were added to a mixture of 400µl L10 and 40µl SC and ss-NA was bound during a 10 min. incubation at room temperature. The tube was subsequently centrifuged (15 sec. at approx. 10.000 x g), and the supernatant was discarded (by suction). The resulting pellet was subsequently washed twice with 1 ml of L2, twice with 1 ml ethanol 70% (vol/vol) and once with 1 ml acetone. The silica pellet was dried (10 min. at 56°C with open lid in an Eppendorf heating block) and eluted in 50µl TE buffer (10 min. at 56°C; closed lid). After centrifugation (2 min. at approx. 10.000 x g) the supernatant contains the ss-NA fraction.

To recover ds-NA forms (protocol R-pellet) from the initial silica-pellet, the remaining supernatant was discarded, and the silica pellet was washed twice with L11 to remove unbound ss-NA. The resulting silica pellet was subsequently washed twice with L2, twice with ethanol 70%, once with acetone, dried and eluted as described above. After centrifugation (2 min. at approx. 10.000 x g) the supernatant contains the ds-NA fraction.

In the complete procedure (which takes about one hour) for fractionation of NA by protocol R, only two Eppendorf tubes are used.

Fractionation of genomic DNA and ss-NA.

Due to trapping of ss-NA into high-molecular-weight genomic DNA, protocol R as described above gives only low yields of ss-NA. This can be circumvented by first isolating total NA by protocol Y/D (11), which causes some shearing of the high-molecular-weight genomic DNA, sufficient enough to prevent trapping of the ss-NA. Total NA thus purified can subsequently be used as input for protocol R.

Gel electrophoresis.

In all experiments, NA was electrophoresed (8 to 10 V/cm) through neutral agarose slab gels containing ethidiumbromide (1µg/ml) in the buffer system (40mM TRIS-20 mM sodium acetate-2mM EDTA adjusted to pH 7.7 with acetic acid; ethidium bromide was added to a concentration of 1µg/ml of buffer) described by Aaij and Borst (14).

Hybridization.

DNA fragments were transferred to nitrocellulose filters by the procedure of Southern (15) and hybridized with [alpha-^{32}P]dCTP labelled pHC624 (16) prepared by random labeling (Boehringer, Germany). Hybridization conditions were as described previously (12).

RESULTS

Comparison of different GuSCN-containing lysisbuffers with respect to the binding of different NA-types to silica particles revealed that only doublestranded forms were bound when using L11 (which is about 100 mM for EDTA) as binding buffer; on the other hand both double- and single-stranded forms were bound in binding buffer L6 (which is about 20 mM for EDTA) (Table 1). These observations formed the basis for the development of a protocol (Protocol R) for the fractionation of single-stranded nucleic acids and double-stranded nucleic acids (Fig. 1)

Once double-stranded nucleic acid is bound by silica particles in L11, a brief centrifugation will separate the silica/ds-NA pellet from the supernatant containing the single-stranded forms. Addition of this supernatant to a mixture of silica particles and binding buffer L10 (which is about 250 mM for Mg^{2+}) the binding of single-stranded nucleic acids to the silica particles is restored. Double-stranded and single-stranded forms can subsequently be purified by washing and eluting the silica-NA complexes (protocol R). Double-stranded nucleic acid is recovered from the initial silica-

pellet (protocol R-pellet), whereas single-stranded forms are recovered from the initial supernatant (protocol R-sup).

For optimization of protocol R we performed reconstruction experiments in which previously purified or commercially available, nucleic acids were mixed and subsequently fractionated by protocol R.

Fractionation of a mixture of double-stranded DNA and single-stranded DNA.

The fractionation of a ds-DNA/ss-DNA mixture, into double stranded- and single stranded forms is shown in Figure 2. The recovery estimated from the band intensity of the ethidium bromide stained gel for ss-DNA was about 50%, the estimated recovery of ds-DNA in the range of 500 bp to 4,6 kb was 80%-90% [similar recoveries were obtained for ds-DNA fragments in the range of 100-500 bp (not shown)], larger fragments were significantly sheared as noted before (11). At the level of detection by UV-illumination, fractionation into ds- and ss-forms was complete.

Fractionation of a mixture of double-stranded RNA and single-stranded RNA.

Figure 3 shows the fractionation of a mixture of ds-RNA (human Rotavirus genome segments 1-11; for review see 14) and ss-RNA (phage MS2 RNA) into double stranded- and single stranded forms. The estimated recovery of ds-RNA and ss-RNA was at least 80%. At the level of detection by UV-illumination, fractionation into ds- and ss-forms was complete.

Fractionation of a mixture of double-stranded DNA and single-stranded RNA.

In Figure 4 it is shown that ds-DNA can also efficiently be separated from ss-RNA.

Again are the recoveries for both fractions at least 80%. Similar results were obtained when E.coli rRNA (23S and 16S) was used as ss-RNA input (not shown).

In the experiments described above, fractionation of ds- and ss-NA forms (as judged by visual inspection of band intensities after ethidiumbromide staining and UV illumination) appeared to be complete. In order to establish the performance of the fractionation procedure for a mixture of ds-DNA and ss-RNA into ss- and ds-forms, NA purified by protocol R-sup from such a mixture was studied by Southern blotting and hybridization with a ^{32}P-labelled DNA probe, homologous to the ds-DNA used as input for fractionation. This experiment revealed that the ss-NA fraction contained less than 0,1% of the ds-DNA input (figure 5).

Fractionation of a mixture of genomic DNA and single-stranded RNA.

When we investigated the separation of high-molecular-weight (genomic) dsDNA and ss-RNA by direct fractionation using E. coli as input for protocol R, it appeared that the ds-DNA fraction was heavily contaminated with rRNA (Fig. 6, lanes 6 and 7), and ss-RNA recovery was low (Fig. 6, lanes 8 and 9). This was likely due to trapping of RNA into high-molecular-weight (genomic) ds-DNA when silica/NA complexes were formed. On the other hand no genomic DNA was observed in the ss-RNA fraction. Total nucleic acid, which was first isolated using the standard protocol Y/D (11), and hereafter used as input material in protocol R showed significantly higher recoveries of the ss-RNA fraction (Fig. 6, lanes 2 and 5).

Amplifications

MATERIALS AND METHODS

Source of nucleic acids.

HIV-1 RNA was isolated from a virus culture (23), phage MS-2 RNA was purchased from Boehringer (Mannheim, Germany) and the 7.5 Kb Poly(A)Tailed RNA and the 100 bp ladder used as a marker were purchased from Life Technologies (Gaithersburg, Maryland, USA). The PCR TA3 cloning vector was obtained from Promega (Madison, USA). Plasmids 5' NOT Hxb_2ENN (24) [containing the GAG and POL genes of HIV-1 from nucleotide 638-4647] and 168.1 RTN (24) [containing the ENV gene of HIV-1 from nucleotide 5674-8474] were purified as described by Ish-Horowicz and Burke (13) followed by protocol R-pellet as described in the examples. The plasmid pHCrec used as a positive control in the PCR experiments was made by a low annealing PCR on lambda DNA (Boehringer) using PCR primer RB 8 (see below). The discrete PCR products were purified using protocol Y/D (11) and subsequently cloned in a PCR III vector (Invitrogen) . The revealing plasmid, pHCrec with a approximately 600 bp insert was subsequently purified from E.coli HB101 as described by Ish-Horowicz and Burke (13) followed by column chromotography with Sepharose CL2B (Pharmacia, Inc. Uppsala, Sweden).

Chemicals and enzymes

EDTA, KCl, $MgCl_2.6H_2O$, NaCl and tri-Sodium citrate dihydrate were obtained from Merck (Darmstadt, Germany).TRIS and BSA were obtained from Boehringer (Mannheim, Germany). Triton X-100 was obtained from Packard (Packard Instruments Co., Inc., Downers, Ill, USA). Sodium Dodecylsulfate (SDS) was obtained from Serva (Heidelberg, Germany).

The dNTP's and Dextran Sulphate were obtained from Pharmacia (Uppsala, Sweden).

The chemicals used in protocol R have been described herein.

Reverse transcriptase SuperScript II was purchased from Life Technologies (Gaithersburg, Maryland, USA). DNA polymerase Sequenase 2 was obtained from Amersham (United Kingdom). Ampli-Taq DNA polymerase was obtained from Perkin Elmer (Norwalk, USA). RNAse H was obtained from Boehringer (Mannheim, Germany). Salmon sperm DNA was obtained from Sigma (St. Louis, USA).

Composition of buffers and solutions.

The preparation of the buffers used in protocol R have been described herein, except that the lysis buffer and washing buffers (L10, L11, and L2) used in protocol R for the isolation of nucleic acids were filtered through a column packed with Diatoms (11) in order to remove any endogenous nucleic acids in the lysis buffer and washing buffers.

The 10 x reverse transcription buffer (CMB1) consists of 100 mM Tris.HCl (pH 8.5), 500 mM KCl and 1% Triton X-100.

The 10 x PCR buffer consists of 500 mM Tris.HCl (pH 8.3), 200 mM KCl and 1 mg/ml BSA.

The elution buffer Tris/EDTA (TE, pH 8.0) consists of 10 mM Tris.HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

Oligonucleotides.

The first strand primer **TAG 20**:

5'GACAGAATGCCGAAATGACCCCNNNNNG3'

The second strand primer **TAG 7**:

5'DIG-GACAGAATGCCGAAATGANNNNNG3'

The PCR primer RB 8:

5'GACAGAATGCCGAAATGA3'

underlined: PCR motif
bold: motif for direct sequencing
N= A, T, C, or G

Protocol for first strand synthesis.

ss-RNA, present in the commercially available reverse transcriptases, appeared to produce unwanted side products when used in first strand synthesis. To overcome this problem reverse transcriptase was first pretreated in a mixture for cDNA synthesis lacking exogenously added primers:

1 µl SuperScript II (200 U/µl)
1 µl CMB1 (10 x)
0.5 µL $MgCl_2$ (100 mM)
0.4 µL dNTP's (25 µM each)
7.1 µL H_2O
Incubate 15 min. at 37°C

Nucleic acids (20 µl) purified by protocol R-sup were incubated for 5 min. at 60°C and thereafter quenched on ice. Subsequently the following mixture was added:
3 µl CMB1 (10 x)
1 µl TAG 20 (100 ng/µl)
1.5 µL $MgCl_2$ (100 mM)
1.2 µl dNTP's (25 mM each)
3.3 µl H_2O

Finally 10 µl of the preincubated Superscript II (SS II) was added and the resulting mixture was incubated for 30 min. at 42°C.

After the reverse transcription reaction SS II was inactivated by incubating the mixture for 5 min. at 80°C, and the mixture was subsequently cooled down to room temperature. In order to convert the RNA/DNA hybrids into single-stranded cDNA twenty units of RNAse H were added to the mixture and incubated for 60 min. at 37°C. The single-stranded cDNA was subsequently isolated using protocol R-sup. The single-

stranded cDNA was eluted in 40 µl TE and 20 µl was used as input for second strand synthesis.

Protocol for second strand synthesis.

To twenty microliter of single-stranded cDNA, the following mixture was added (on ice):

4 µl CMB1	(10 x)
1 µl TAG 7-DIG*	(100 ng/µl)
2 µl $MgCl_2$	(100 mM)
1.6 µl dNTP's	(25 mM each)
0.2 µl Sequenase 2	(13 U/µl)
11.2 µl H_2O	

The mixture was incubated for 10 min. on ice, and subsequently for 60 min. at 37°C. After the second strand synthesis the double-stranded cDNA was isolated using protocol R-pellet. The double-stranded cDNA was eluted in 40 µl TE. Twently microliter was taken of and 2 µl was used as input for PCR. The remaining 18 µl was stored at -20°C.

Protocol for the polymerase chain reaction.

Two microliters of double-stranded cDNA was added to 48 µl of a PCR mixture consisting of:

18 µl TE	(pH 8.0)
1 µl RB 8	(100 ng/µl)
5 µl PCR buffer	(10 x)
0.9 µl $MgCl_2$	(100 mM)
0.2 µl dNTP's	(100 µM)
0.1 µl dUTP*	(25 µM)
0.3 µl Ampli Taq	(5 U/µl)
22.5 µl H_2O	

After incubation for 5 min. at 95°C the sample was subjected to 45 cycles of amplification in a DNA thermal cycler (type 480; Perkin Elmer Cetus). A cycle consisted of

denaturation for 1 min. at 95°C, annealing for 1 min. at 63°C, and elongation for 2 min. at 72°C. After cycling the sample was incubated for 8 min. at 72°C, and subsequently the temperature was lowered to 4°C. Twentyfive microliter of the PCR product was examined by agarose gel electrophoresis and ethidiumbromide staining. In every experiment TE was used as a negative extraction control and as a negative PCR control.

*Partial substitution of dTTP with dUTP provides a methodology for ensuring that products from previous PCR reactions cannot be reamplified. Products of PCR amplifications will be uracil-containing deoxyribonucleic acids. Possible contaminating PCR products from a previous PCR amplification will be eliminated by excising uracil bases using the enzyme Uracil N-glycosylase (UNG) prior to PCR (25)

Gel electrophoresis.

In all experiments, the nucleic acids were electrophoresed (8 to 10 V/cm) through neutral agarose slab gels containing ethidiumbromide (1 µg/ml) in the buffer system as described by Aaij and Borst (14)

Hybridization.

DNA fragments were detected after Southern blotting (15) by hybridization with ^{32}P-labelled probes representing the entire GAG, POL, and ENV genes of HIV-1 [plasmid 5' NOT Hxb$_2$ENN and plasmid 168 1 RTN](10).

RESULTS

In parallel, 10^5 molecules of HIV-1 RNA (23) and negative controls (TE) were extracted using protocol R-sup. The resulting single-stranded nucleic acids were amplified by the non-selective RT-PCR as disclosed above, resulting in a discrete banding pattern for HIV-1 RNA, and no amplification products in the TE controls (Fig. 8). The variation between the duplicates is a reflection of the non-selectivity of the procedure. As a control for the efficiency of the PCR part of

the procedure we used an input of 0, 6, 60 and 600 molecules of the plasmid pHCrec.

In order to confirm the HIV-origin of the bands visible in figure 8 A, we performed a Southern blot hybridization under high stringency conditions with ^{32}P-labelled probes encompassing almost the entire HIV-1 genome (Fig. 8 B). This experiment showed that most of the bands visible by UV-illumination hybridized with the HIV-1 probe. The bands that did not hybridize with the probe might be homologous to parts of the HIV-1 genome other than those present in the probe or might originate from single-stranded RNA present in the HIV-1 RNA preparation (e.g. cellular mRNA) or ss-RNA present in Superscript II, which was not converted to ds-hybrids during the preincubation of the SuperScript II.

Similar results were obtained with other single-stranded RNAs such as hepatitis C virus RNA, phage MS2 RNA, and the 7.5 Kb Poly(A)-Tailed RNA (results not shown).

It is concluded that the described procedure can be used to amplify single stranded RNA targets (present in e.g. serum) to a series of discrete bands in agarose gels. The discrete bands can be purified from agarose gels, cloned in e.g. a bacterial vector and the clones can subsequently be sequenced. Due to the fact that one of the tagging primers (TAG 20) harbours a sequence motif it is possible to sequence the discrete bands without cloning, after the bands are purified from gel. The method described here is useful in isolating and characterizing unknown sequences present in clinical samples (e.g. viral sequences) or for the amplification of cDNAs from transcripts without having any sequence data.

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NA-type	binding in L6	binding in L11
ds-DNA	+	+
ds-RNA	+	+
ss-DNA	+	-
ss-RNA	+	-

Table 1.

Binding of different NA-types to silica particles in different lysisbuffers; similar results were obtained using diatoms rather than silica particles (data not shown).

CLAIMS

1. A method for separating single stranded nucleic acid material from double stranded nucleic acid material comprising contacting a mixture of the both with a liquid comprising a chaotropic agent and a nucleic acid binding solid phase, whereby the liquid has a composition such that double stranded nucleic acid binds to the solid phase and a substantial amount of single stranded nucleic acid does not and separating the solid phase from the liquid.

2. A method according to claim 1 whereby the liquid comprises at least 100 mM EDTA and comprising a guanidinium salt, preferably guanidinium thiocyanate as a chaotropic agent.

3. A method according to claim 1 or 2, whereby the solid phase is silicium based.

4. A method according to claim 3 whereby the solid phase is silica.

5. A method according to claim 4 whereby the silica is in the form of particles having a size between 0.05 and 500, preferably 0.1 and 200 µm.

6. A method according to anyone of the aforegoing claims whereby the solid phase is separated from the supernatant by centrifugation.

7. A method for isolating single stranded nucleic acid material from a mixture of nucleic acid material, comprising the steps of subjecting the mixture to a method according to anyone of the aforegoing claims and treating the supernatant containing the single stranded nucleic acid material with a second liquid comprising a chaotropic agent and a second nucleic acid binding solid phase, whereby the second liquid has a compositon such that the resulting mixture of supernatant and second liquid allow for binding of the single stranded nucleic acid material to the second solid phase.

8. A method for amplifying single stranded nucl ic acid material comprising the steps of hybridizing the single

stranded nucleic acid with primers and elongating the probes using an enzyme which adds nucleotides to the primer sequence using the hybridized single strand material as a template, whereby at least one primer comprises a random hybridizing sequence and an amplification motif.

9. A method according to claim 8 whereby at least one primer comprising a random hybridization sequence and an amplification sequence further comprises a label.

10. A method according to claim 8 or 9 whereby at least one primer comprising a random hybridizing sequence and an amplification motif further comprises a direct sequencing motif.

11. A method for isolating and amplifying single stranded nucleic acid material originally present in a mixture of nucleic acids comprising subjecting the mixture to a method according to anyone of claims 1-7 followed by subjection of the isolated material to a method according to anyone of claims 8-10.

12. A method according to any one of the aforegoing claims whereby the single stranded nuclec acid material comprises mRNA.

13. A method according to claim 12 whereby the mRNA is converted into cDNA.

14. A method according to anyone of the aforegoing claims comprising a gel electrophoresis step.

15. A method according to anyone of the aforegoing claims followed by a sequencing step.

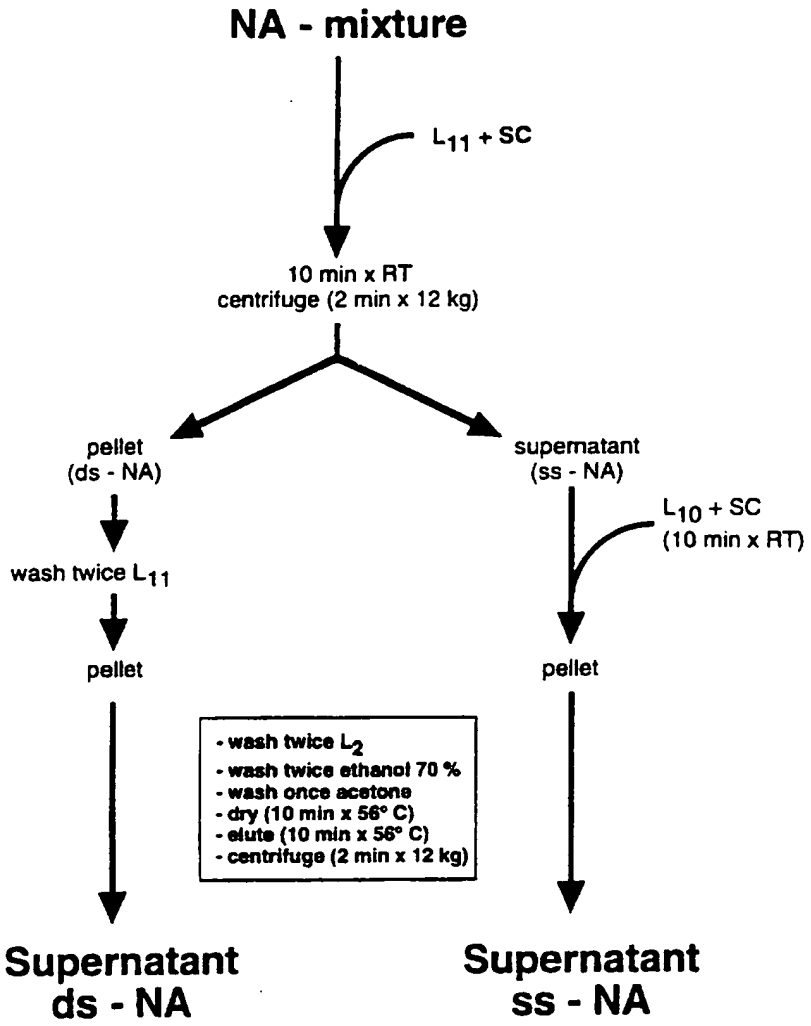


Figure 1. Outline of protocol R.

Recovery of ds-NA takes place from the initial pellet (R-pellet), recovery of ss-NA takes place from the initial supernatant (R-sup). L11, L10, L6 and L2 are GuSCN based-buffers, SC is silica particle suspension. For details see Materials & Methods section.

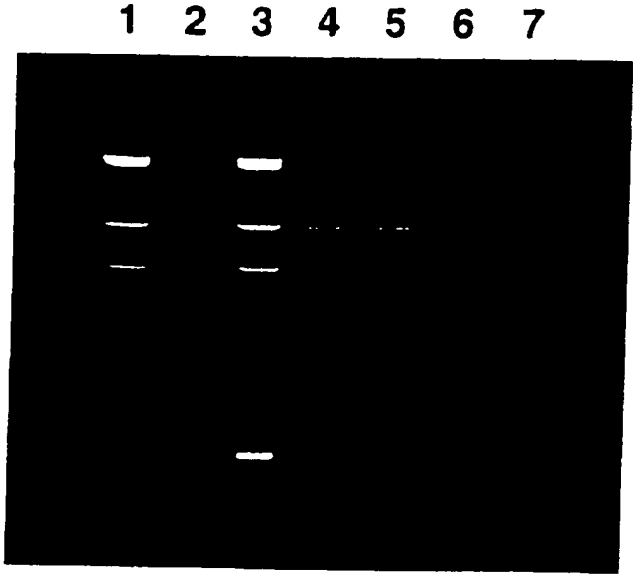


Figure 2. Separation of ds-DNA and ss-DNA.

NA was purified (in duplicate) by protocol R from a mixture of ds-DNA (phage lambda, HindIII digest, 1μg) and ss-DNA (phage M13 DNA, 500ng). Final elutions were in 50μl TE and 25μl were electrophoresed through a 1% agarose gel (containing ethidiumbromide) which was subsequently photographed under UV-illumination. Lane 1, 100% recovery marker for ds-DNA fragments; lane 2, 100% recovery marker ss-DNA; lane 3, 100% recovery marker mixture ds-DNA/ ss-DNA. Lanes 4 and 5, output protocol R-pellet; lanes 6 and 7, output protocol R-sup.

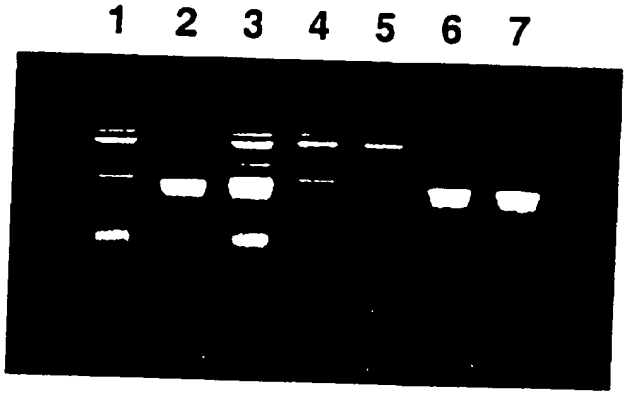


Figure 3. Separation of ds-RNA and ss-RNA.

NA was purified (in duplicate) by protocol R from a mixture of ds-RNA (Rotavirus ds-RNA) and ss-RNA (phage MS2 RNA, 800ng). Final elutions were in 50μl TE and 25μl were electrophoresed through a 1% agarose gel (containing ethidiumbromide) which was subsequently photographed under UV-illumination. Lane 1, 100% recovery marker for ds-RNA fragments; lane 2, 100% recovery marker ss-RNA; lane 3, 100% recovery marker ds-RNA/ss-RNA mixture. Lanes 4 and 5, output protocol R-pellet; lanes 6 and 7, output protocol R-sup.

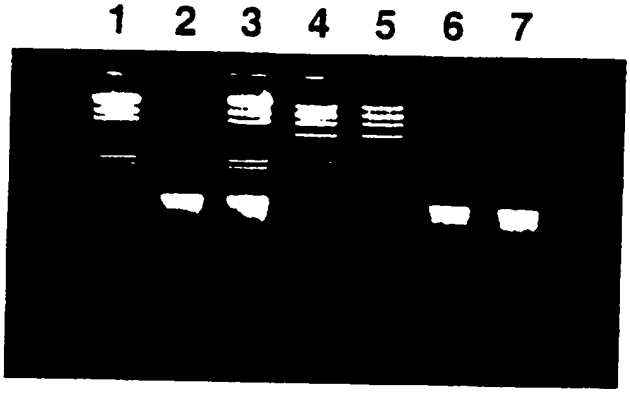


Figure 4. Separation of ds-DNA and ss-RNA.

NA was purified (in duplicate) by protocol R from a mixture of ds-DNA (750 ng phage lambda digested with hindIII) and ss-RNA (phage MS2 RNA, 800ng). Final elutions were in 50μl TE and 25μl were electrophoresed through a 1% agarose gel (containing ethidiumbromide) which was subsequently photographed under UV-illumination. Lane 1, 100% recovery marker for ds-DNA; lane 2, 100% recovery marker for ss-RNA; lane 3, 100% recovery marker for ds-DNA/ss-RNA mixture. Lanes 4 and 5, output protocol R-pellet; lanes 6 and 7, output protocol R-sup.

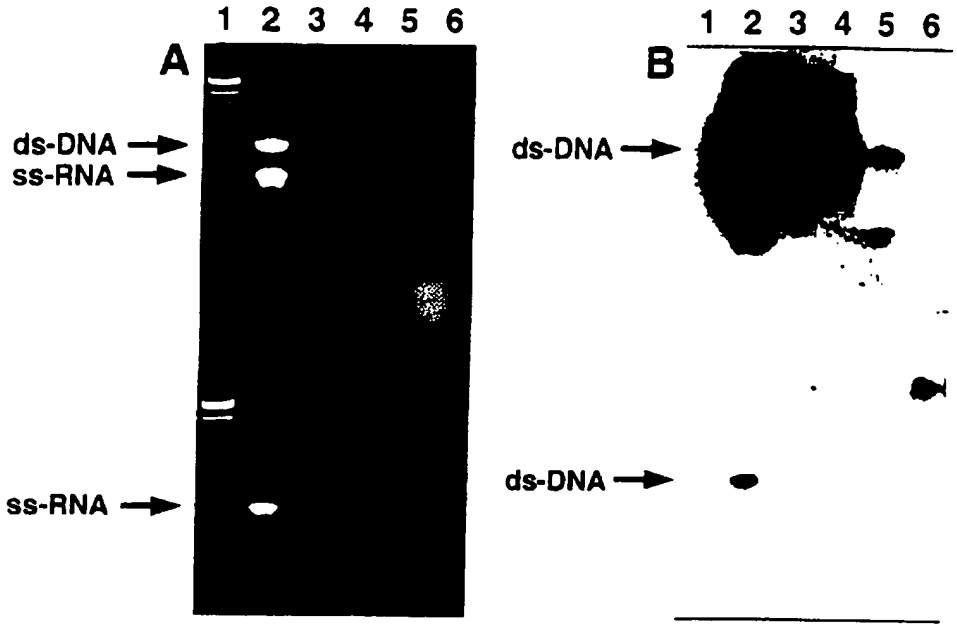


Figure 5. Separation of ds-DNA and ssRNA.

NA was purified by protocol R-sup from a mixture of ds-DNA (1000 ng linearized pHC624, 2kb) and ss-RNA (phage MS2 RNA, 800ng). Final elution was in 50μl TE, and 25μl or tenfold serial dilutions of the ss-NA fraction were electrophoresed through a 1% agarose gel (containing ethidiumbromide) which was subsequently photographed under UV-illumination.

Panel A: Upper row: lane 1, HindIII digested phage lamdba DNA; lane 2, 100% recovery marker for ds-DNA and ss-RNA and serial tenfold dilutions thereof (lanes 3-6) . Bottom row, output of protocol R-sup (lane 2) and tenfold serial dilutions (lanes 3-6).

Panel B: Ds-DNA was subsequently transferred to a nitrocellulose filter and hybridized with a ^{32}P-labelled probe homologous to input ds-DNA.

ds-DNA and ss-RNA are indicated.

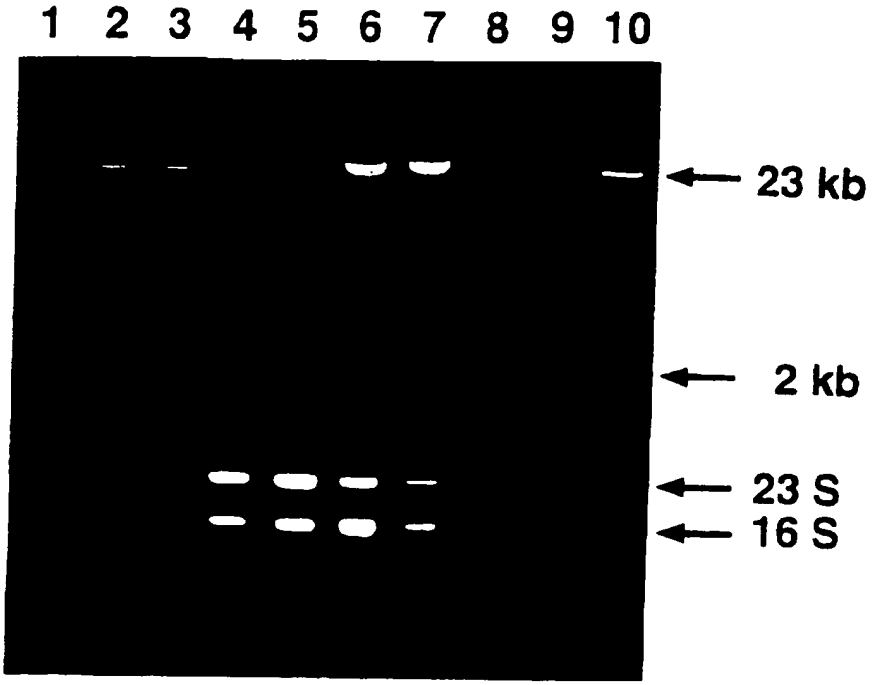


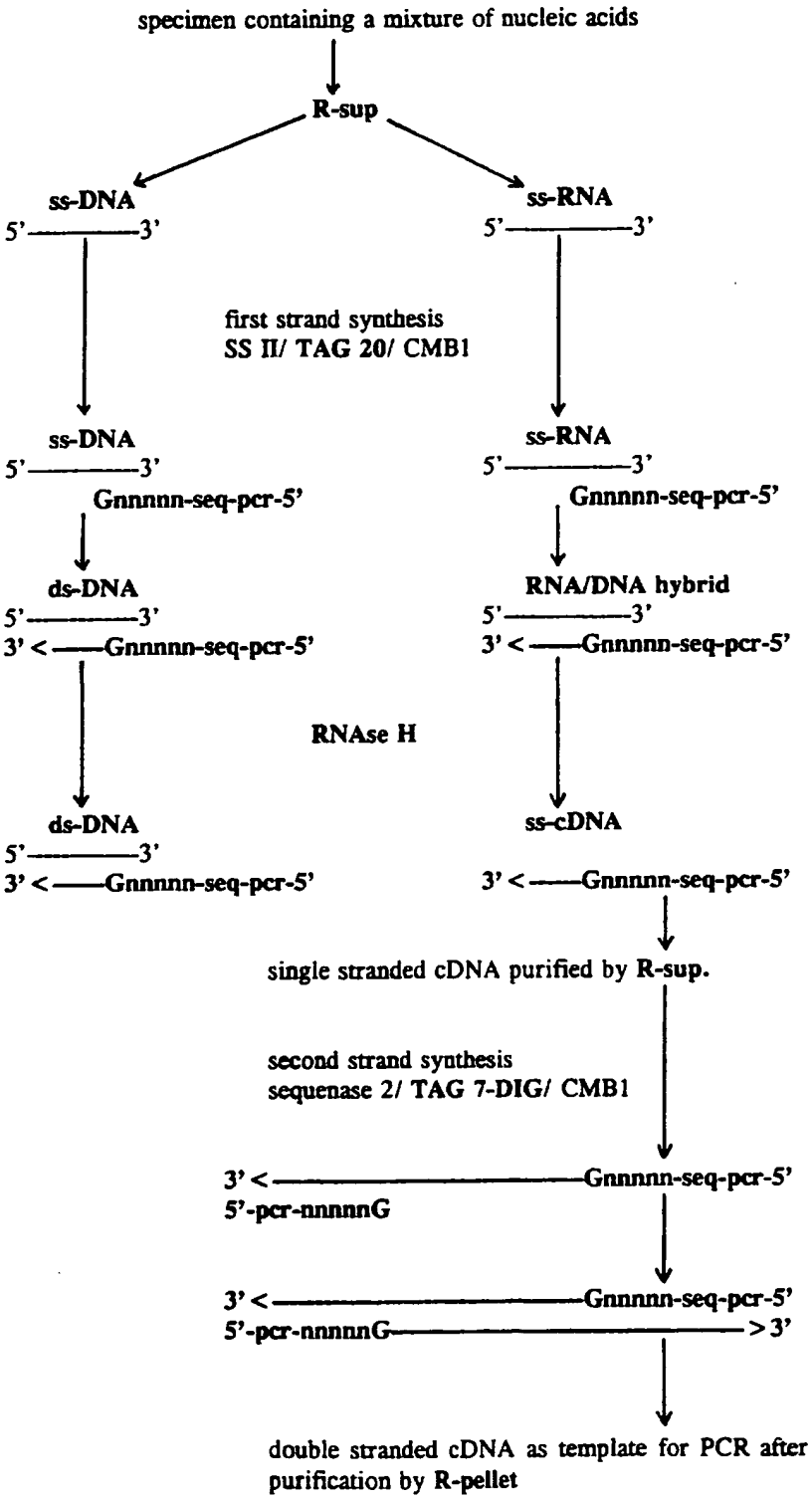
Figure 6. Separation of genomic DNA from ss-RNA.

How to deal with trapping of ss-RNA. E. coli bacteria were directly used as inputmaterial for duplicate extractions by protocol R (lanes 6 and 7, R-pellet; lanes 8 and 9, R-sup). Alternatively, total NA was first purified by protocol Y using diatoms as NA-carrier (which causes shearing of genomic DNA). The purified nucleic acids were subsequently used as input for protocol R (lanes 2 and 3, R-pellet; lanes 4 and 5, R-sup). Final elutions were in 50μl TE and 25μl were electrophoresed through a 1% agarose gel (containing ethidiumbromide) which was subsequently photographed under UV-illumination.

Markerlanes 1 and 10 500 ng phage lambda DNA, HindIII digested).

23S and 16S rRNA, and ds-DNA molecular weight markers (23kb and 2.0 kb) are indicated.

Figure 7 Outline of the procedure



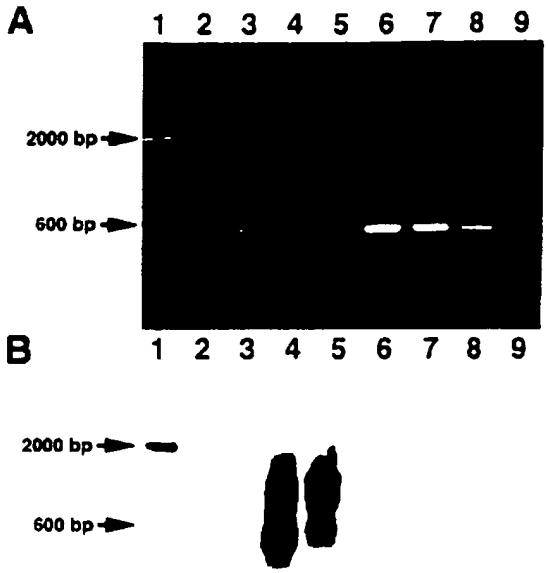


Figure 8

Single-stranded nucleic acid was purified from samples containing HIV-1 RNA and TE (negative control) by protocol R-sup. and subsequently amplified with the non-selective RT-PCR.

Panel A: lane 1, 100 bp DNA ladder; lanes 2 and 3 negative extraction controls; lanes 4 and 5 non-selectivily amplified HIV-1 RNA; lanes 6, 7, 8 and 9 600, 60, 6 and 0 molecules resp. of pHCrec (positive PCR control).

Panel B: Southern blot hybridization with ^{32}P-labelled HIV-1 probes (containing the GAG, POL and ENV genes of HIV-1) of the samples shown in panel A. After overnight hybridization at 65 °C in 6 x SSC, 0.1 % SDS, 10 % Dextran Sulphate and 50 µg/ml salmon sperm DNA, the filter was subsequently washed under high stringency conditions with 0.1 SSC/0.1% SDS at 65°C, and autoradiographed on X-ray film for two hrs. at -70°C. This experiment showed that most of the bands visible on the ethidiumbromide stained agarose gel originated from the HIV-1 genome.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00063

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H1/08 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 04140 A (AKZO NOBEL NV ;BOOM WILLEM R (NL); ADRIAANSE HENRIETTE M A (NL); K) 9 February 1995 see the whole document ---	1-7, 11-15
Y	WO 95 34569 A (INVITEK GMBH ;HILLEBRAND TIMO (DE); BENDZKO PETER (DE); PETERS LAR) 21 December 1995 see page 6, paragraph 3 ---	1-7, 11-15
Y	EP 0 389 063 A (AKZO NV) 26 September 1990 cited in the application see the whole document ---	1-7, 11-15
A	US 5 075 430 A (LITTLE MICHAEL C) 24 December 1991 see the whole document ---	1-7, 11-15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 June 1997	31. 07. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	OSBORNE, H

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00063

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 155 018 A (GILLESPIE DAVID ET AL) 13 October 1992 see the whole document	1-7, 11-15
A	WO 95 06652 A (PROMEGA CORP) 9 March 1995 see the whole document	1-7, 11-15
A	EP 0 691 148 A (PERKIN ELMER CORP) 10 January 1996 see the whole document	1,11-15
A	WO 95 21849 A (QIAGEN GMBH ;BASTIAN HELGE (DE); GAUCH SIMONE (DE); COLPAN METIN () 17 August 1995 see the whole document	1,11-15

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internation pplication No.
PCT/NL 97/00063

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-7 and partially 11-15: A method for the separation of single and double stranded nucleic acids in a mixture
2. Claims 8-10 and partially 11-15: A method for the amplification of single stranded nucleic acids

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims: 1-7 and partially 11-15

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No PCT/NL 97/00063

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9504140 A	09-02-95	AU 7375794 A	28-02-95
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